# Improved 2D nano LC/MS for proteomics applications – A comparison on yeast

## Application

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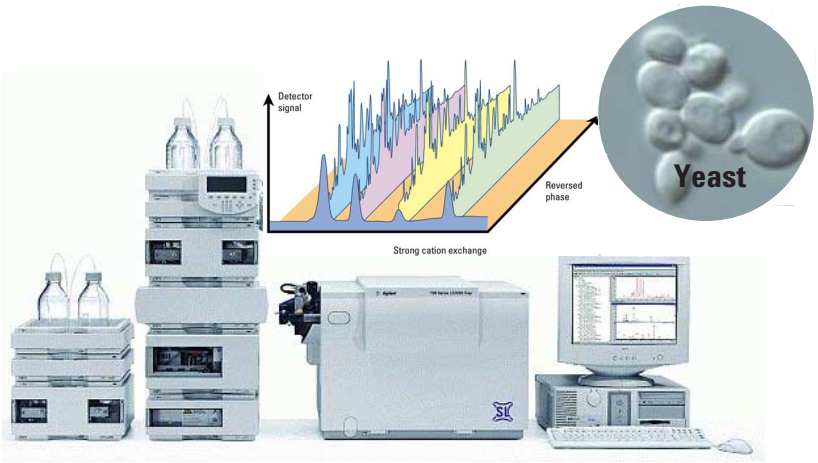


**Abstract**

The most frequently used method for protein identification with two-dimensional LC/MS utilizes the elution of digest peptides from a strong cation exchange column by an injected salt step gradient of increasing salt concentration with a subsequent reversed phase separation. However, in this approach the ion exchange chromatography does not produce its full performance. To improve the SCX chromatography performance a new method was developed, which works with a semi-continuous pumped salt gradient. This Application Note describes the improved method for two-dimensional nano LC/MS. To show the full performance of the method a complex tryptic digest of the yeast proteome was analyzed. The results obtained with the developed method are compared to the formerly used injection method as well as to an off-line 2D LC method.

Agilent Technologies

## Introduction

In order to identify proteins from a complex mixture of 5 x $10^3$ to 5 x $10^4$ with a dynamic range of at least $10^5$ it is crucial to develop technologies with extremely good resolving power on one hand and with extraordinary sensitivity on the other hand. Today, the most frequently used approach for separation of peptides from protein digests in complex proteomics applications by HPLC is two-dimensional nano LC/MS. In this approach a strong cation exchange (SCX) column for the first dimension is combined with a reversed phase (RP) column in the second dimension[1,2]. In the first attempts the sample peptides bound on the SCX columns are eluted with injected salt solution plugs of increasing concentration, trapped on a short enrichment column and subsequently analyzed on a nano RP column. This methodology is capable to deliver valuable results for proteomics research. For instance, there are examples for the elucidation of the yeast proteome[3] and the proteome of other microorganisms[4]. Even the detection of a subproteome consisting of a few proteins in a background of several hundred is possible[5,6]. However, the injected volume of salt solution is not optimized to the SCX column and the SCX column is working far away from its equilibrium. This "not optimized" state of this method is the reason for peptide distribution over more than one fraction. This can dilute them below their detection level or suppress their ionization in nano electrospray by higher abundant peptides in the mass spectrometric analysis.

To overcome these limitations an improved method for online 2D LC was developed. In this method the optimized semi-continuous salt solution gradient for the elution of the peptides from the SCX column is delivered very precisely with an Agilent 1100 Series capillary pump. The SCX column was always kept under conditions very close to the equilibrium. The eluted peptides are trapped rotatory on two enrichment columns and are subjected to reversed phase separation followed by MS/MS analysis. The principle of this method is shown in figure 1. This Application Note explains the improved method for two-dimensional nano LC/MS. To show the full performance of the method a complex tryptic digest of the yeast proteome was analyzed. The results obtained with the new method are compared to the currently used method and also to an offline 2D LC method.

## Experimental

### Equipment

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent micro 2-position/10-port switching valve box with holder
- Agilent 1100 Series capillary pump
- Agilent 1100 Series LC/MSD Trap XCT with orthogonal nanospray ion source

The system set-up is based on the Agilent Nanoflow Proteomics Solution[7].

Software:

- ChemStation A10.01
- Spectrum Mill MS Proteomics Workbench[8]

Columns:

- Agilent BioSCX Series II, 0.30 x 35 mm, 3.5 µm particles
- ZORBAX 300SB C18, 75 µm x 150 mm, 3.5 µm particles
- ZORBAX 300SB C18, 0.3 x 5 mm, 5 µm particles

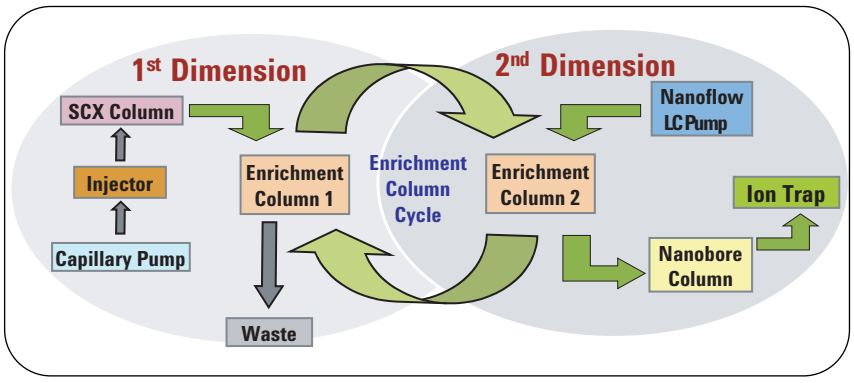


**Figure 1**
**Principle of two-dimensional nano LC-MS/MS with semi-continous-gradient**

The micro 2-position/10-port valve included in the LC-System (figure 2) is connected with two enrichment columns. This valve is also connected directly to the nanoflow pump and to the nanocolumn. The second pump used in the system, the capillary pump, is connected to the micro 2-position/6-port valve in the 1100 Series micro well-plate autosampler, which is also connected with the micro 10-port valve (figure 3). The sample peptides retained on the SCX column are eluted stepwise with a semi-continuous salt solution gradient pumped by the 1100 Series capillary pump and subsequently trapped on the enrichment column currently inline with the SCX column (figure 3A-1). Each step of the semi-continuous salt solution gradient starts with the end concentration of the foregoing step and ends with the starting concentration of the following step. After each step the SCX column is bypassed by switching the micro valve in the 1100 Series autosampler (figure 3A-2) to retain the current salt concentration in the column. In this state the enrichment column is still inline with the capillary pump, which starts to pump water to wash out salt residues from the capillaries and the enrichment column prior to the RP separation and the MS analysis. The salt-free enrichment column is then switched into the nanoflow path and exchanged with the second enrichment column also located at the micro 10-port valve and the cycle starts over again (figure 3 B).

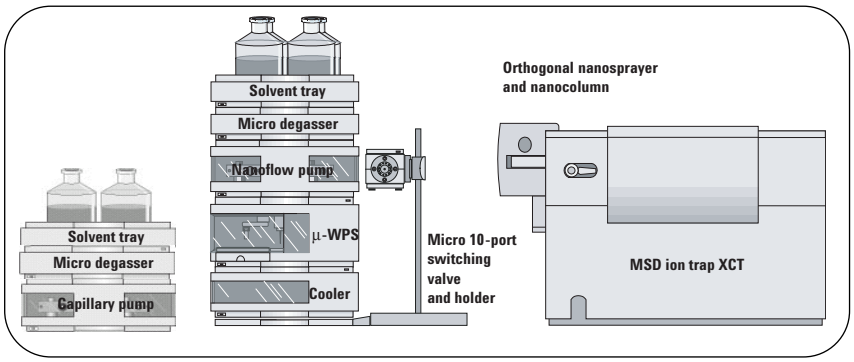


**Figure 2**
**Nano LC/MS system for online 2D LC/MS with semi-continuous gradient for proteomics applications**

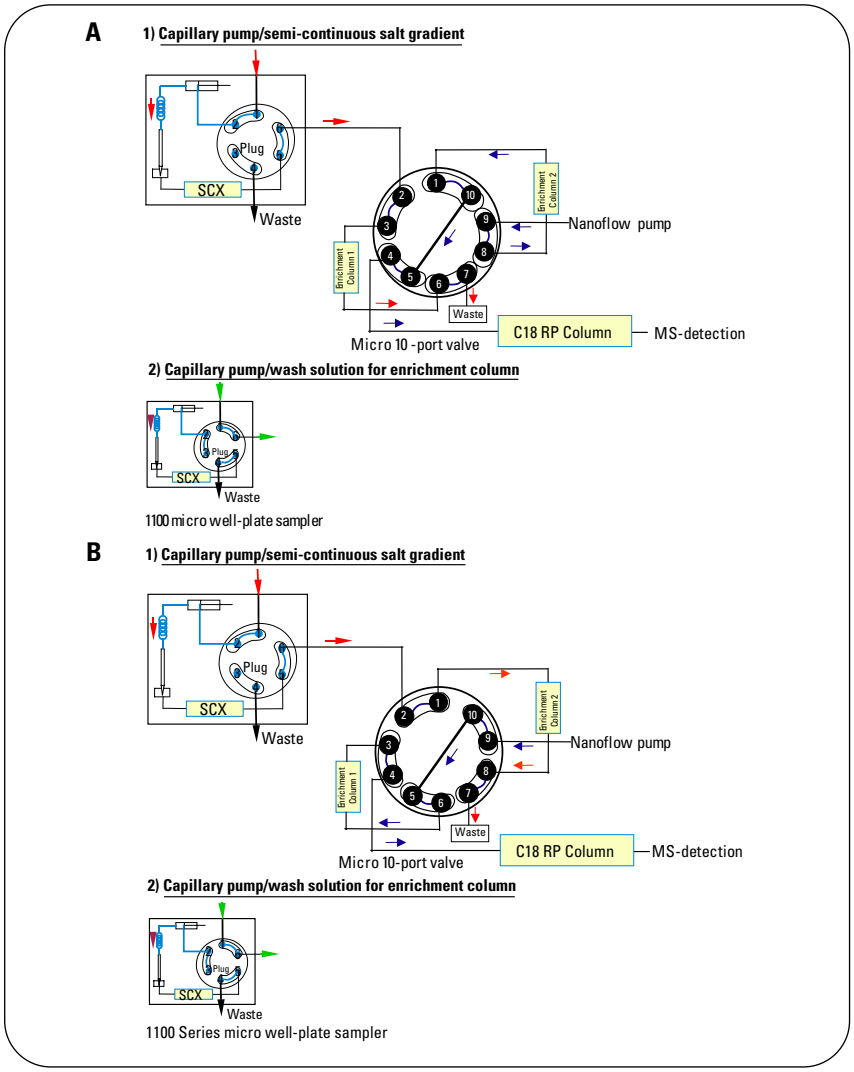


**Figure 3**
**Flow diagram for semi-continuous gradient in online nano 2D LC**
**A. 1) Continuous salt elution from SCX column on enrichment column.**
**2) Bypassing of SCX column, washing of enrichment column 1 and analysis of peptides from enrichment column 2**
**B. 1) Continuous salt elution from SCX column on enrichment column 2**
**2) Bypassing of SCX column, washing of enrichment column 2 and analysis of peptides from enrichment column 1**

| Control of pumps and valves for semi-continuous 2D LC | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Nanoflow pump** | | | | | | | | | | | | | | | | | | | | | |
| Time [min] | 0.00 | 10.00 | 70.00 | 70.01 | 85.00 | 145.00 | 145.01 | 160.00 | 220.00 | 220.01 | 235.00 | 295.00 | 295.01 | 310.00 | 370.00 | 370.01 | 385.00 | | | | |
| % Solvent B | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | | | | |
| Time [min] | 445.00 | 445.01 | 460.00 | 520.00 | 520.01 | 535.00 | 595.00 | 595.01 | 610.00 | 670.00 | 670.01 | 685.00 | 745.00 | 745.01 | 760.00 | 820.00 | 820.01 | | | | |
| % Solvent B | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | 5.00 | 65.00 | 5.00 | | | | |
| **Capillary pump** | | | | | | | | | | | | | | | | | | | | | |
| Time [min] | 0.00 | 15.00 | 30.00 | 30.01 | 90.00 | 90.01 | 105.00 | 105.01 | 165.00 | 165.01 | 180.00 | 180.01 | 240.00 | 240.01 | 255.00 | 255.01 | 315.00 | 315.01 | 330.00 | 330.01 | |
| % Solvent B | 0.00 | 0.00 | 2.50 | 0.00 | 0.00 | 2.50 | 5.00 | 0.00 | 0.00 | 5.00 | 7.50 | 0.00 | 0.00 | 7.50 | 10.00 | 0.00 | 0.00 | 10.00 | 15.00 | 0.00 | |
| Time [min] | 390.00 | 390.01 | 405.00 | 405.01 | 465.00 | 465.01 | 480.00 | 480.01 | 540.00 | 540.01 | 555.00 | 555.01 | 615.00 | 615.01 | 630.00 | 630.01 | 690.00 | 690.01 | 705.00 | 705.01 | |
| % Solvent B | 0.00 | 15.00 | 20.00 | 0.00 | 0.00 | 20.00 | 30.00 | 0.00 | 0.00 | 30.00 | 50.00 | 0.00 | 0.00 | 50.00 | 100.00 | 0.00 | 0.00 | 100.00 | 100.00 | 0.00 | |
| **10-Port valve** | | | | | | | | | | | | | | | | | | | | | |
| Switch Position | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | | | | | | | | | |
| Time [min] | 0.00 | 10.00 | 85.00 | 160.00 | 235.00 | 310.00 | 385.00 | 460.00 | 535.00 | 610.00 | 690.00 | 760.00 | | | | | | | | | |
| **Micro well-plate autosampler 6-port valve** | | | | | | | | | | | | | | | | | | | | | |
| Mainpass time [min] | 0.00 | 90.00 | 165.00 | 240.00 | 315.00 | 390.00 | 465.00 | 540.00 | 615.00 | 690.00 | 765.00 | | | | | | | | | | |
| Bypass time [min] | 30.00 | 105.00 | 180.00 | 255.00 | 330.00 | 405.00 | 480.00 | 555.00 | 630.00 | 705.00 | | | | | | | | | | | |

**Table 1**
**Gradient settings for the SCX- and RP-chromatograpy, switching points for autosampler 6-port valve and enrichment column 10-port valve.**

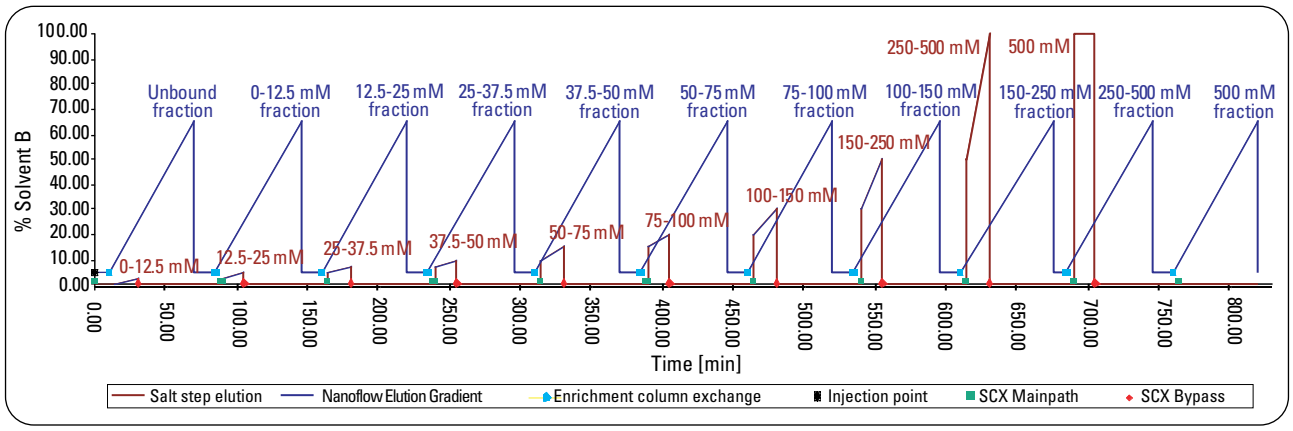


**Figure 4**
**Control method of the 2D online semi-continous step gradient LC. Blue: Reversed Phase gradient. Brown: semi-continuous salt solution gradient.**

## Chromatographic method

For the chromatographic separation in the first- and second dimension it is necessary to set-up two different methods, one for the SCX chromatography and another one for the RP separation. The semi-continuous salt solution gradient for the elution of the peptides from the SCX column is delivered from the Agilent 1100 Series capillary pump and the gradient for the RP separation is delivered from the 1100 Series nanoflow pump (figure 4). The nanoflow gradient starts with 5 % acetonitrile and increases up to 65 % acetonitrile with a slope of 1 %/min for each RP analysis. The salt gradient is pumped in steps beginning at 0 % to 2.5% of a 500 mM NaCl solution for the fist step. The following steps start with the end concentration of the foregoing step and end with the starting concentration of the following step. The salt solution gradient is developed for 15 minutes in each step. Prior to the washing step the SCX column is switched to bypass with the micro 6-port valve in the autosampler to retain the equilibrium condition. Therefore, each step contributes to a quasi-linear salt gradient on the SCX column (figure 4). To get a good separation for the majority of peptides eluting at lower salt concentration the slope is shallower in this area and steeper in the area of higher salt concentrations. The micro 6-port valve in the autosampler switches the SCX column into the salt solution flow at the beginning of each

step and switches the SCX column to bypass at the and of each salt step (figure 4). At the starting point of each RP analysis cycle the charged enrichment column is exchanged against the empty one by a switch of the micro 10-port valve (figure 4). The detailed gradient settings for the SCX and the RP chromatography, the valve switching points for the autosampler micro 6-port valve and the micro 10-port valve are outlined in table 1. The settings of the nanoflow- and capillary pump and the MSD ion trap XCT are summarized in table 2.

### Sample Preparation

Lyophilized yeast cells (*Saccharomyces cerevisiae*), resuspended in cooled 50 mM $NH_4HCO_3$ containing 8M urea were disrupted in a bead beater (0.5 mm glass beads). After centrifugation to remove cell debris, proteins in the supernatant were reduced with 1 mM DTT at 37 °C for 1 h, alkylated in the dark with 10 mM iodoacetamide for 30 min at RT, ultrafiltrated for buffer exchange and tryptically digested with TPCK trypsin at 37 °C for 16 h. Finally the sample was lyophilized in a SpeedVac and dissolved in 5 % AcN, 0.03 % formic acid prior to analysis.

**Chromatographic conditions:**

**Nanoflow pump**

Solvents: A = Water + 0.1 % FA, B = AcN + 0.1 % FA.
Primary flow: 200-500 µL/min
Column flow: 300 nL/min.
Stop time 825 min, Post time 15 min.

**Capillary pump**

Solvents: A = Water + 3 % AcN + 0.1 % FA, B = 500 mM NaCl + 3 % AcN + 0.1 % FA.
Primary flow: 500-800 µL/min
Column flow: 10 µL/min.

**Trap XCT MS conditions**

Ionization mode: positive nanoelectrospray with Agilent orthogonal source
Drying gas flow: 5 L/min
Drying gas temperature: 300 °C
Vcap: typically 1800-2000 V
Skim 1: 30 V
Capillary exit offset: 75 V
Trap drive: 85 V
Averages: 1 or 2

ICC: On
Maximum accumulation time: 150 ms
Smart Target: 125,000
MS Scan range: 300-2200
Automatic MS/MS:
Peptide scan mode
Number of parents: 3 or 4
Averages: 2
Fragmentation amplitude: 1.3 V
SmartFrag: On, 30-200%
Active Exclusion: On, 2 spectra, 1 min

**Table 2**
**Settings of the nanoflow- and capillary pump and the MSD ion trap XCT.**

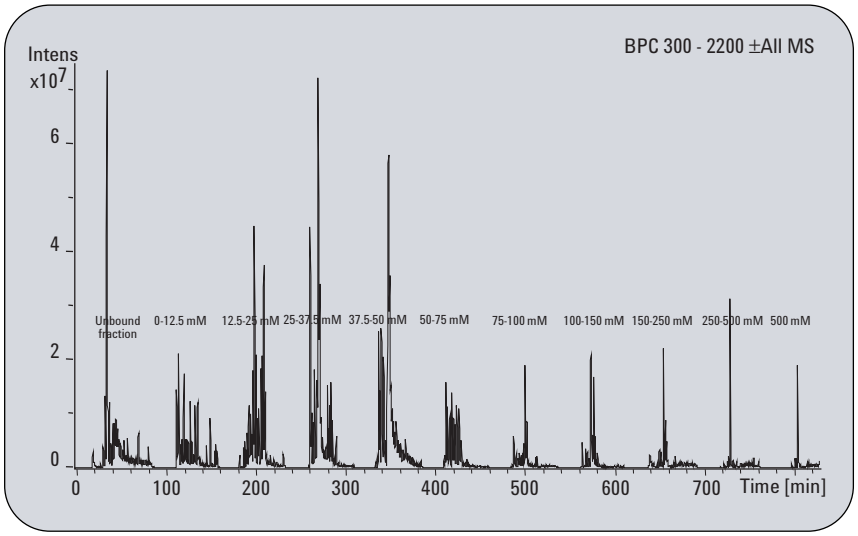


**Figure 5**
**Base peak chromatogram obtained from separation of a yeast proteome digest with online 2D LC working with semi-continuous salt gradient and subsequent nano reversed phase chromatography**

## Results and discussion

For the 2D LC method, which works with an injected salt step gradient, the obtained peptide MS/MS spectra are stored in different data files because for each injected salt concentration step and the subsequent RP separation a new run is started. Compared to this electronic filing the new method saves all the MS/MS data obtained after PR separation for the pumped semi-continuous salt step gradient into one data file. Therefore, the whole 820 minute run can be displayed in one chromatogram (figure 5). As an example, a whole yeast proteome digest was separated with this technique. The obtained base peak chromatogram (figure 5) indicates that, besides the peptides in the unbound fraction, the majority of the peptides are eluted in the semi-continuous salt gradient up to a salt concentration of 100 mM. Therefore, this region was divided into smaller salt elution steps and the region from 100 mM up to 500 mM into larger salt elution steps. There are only a few remaining peptides eluted at the high salt concentrations, which are bound very strongly to the ion

exchanger resin. Figure 6 shows the magnified base peak chromatograms obtained after RP separation for all semi-continuous salt elution steps up to 100 mM. The peptides resulting to each salt step are separated in 30 minutes in subsequent RP gradient run. In the same time frame the associated MS/MS spectra necessary for the database search are acquired (figure 7). During the entire runtime more than 30,000 MS/MS spectra were acquired. From those spectra only the peptide MS/MS spectra were extracted with the spectra extractor of the Spectrum Mill software within a few minutes. After the subsequent search in the NCBI database and validation of the obtained protein hits 122 proteins were identified confidentially. The 21 top score proteins are shown in table 3. They are identified with a very high and confident score and number of peptides and with a sufficient number of acquired spectra and sequence coverage. This is also correct for the proteins at the end of this list. The proteins hit numbers 120-122 are also included in table 3. To emphasise the high quality and confidence of the acquired MS/MS spectra and the reliability of the according protein hits obtained from the database search the spectra for the second and last hits in the list were checked manually. The tryptic peptide (GVE)VVLPVDFIIADAFSADA(NTK) according to the top score protein phosphoglycerat kinase shows a complete fragmentation pattern with all y- and b-series ions (figure 8A). The same was found for the fragmentation pattern of the tryptic peptide (SQ)LAQQIQAR from protein hit number 120 poly-adenylat-binding protein (figure 8B).

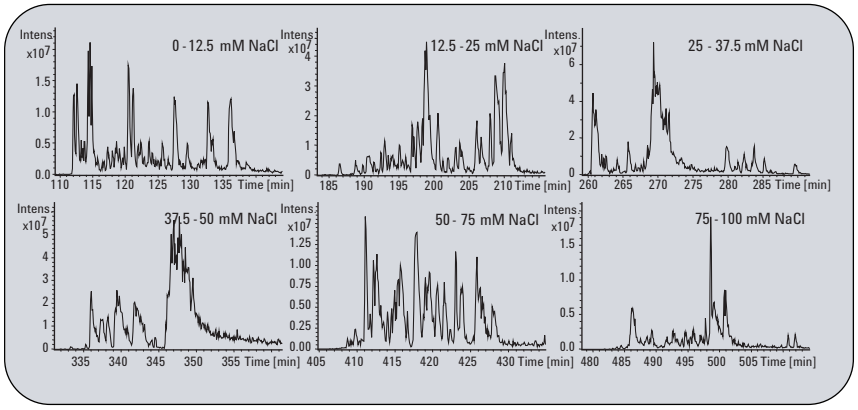


**Figure 6**
**Base peak chromatograms obtained after RP separation for the semi-continuous salt elution steps up to 100 mM.**

| Hit # | Protein Name | Score | Peptides | Spectra # | % AA Coverage | Protein MW (Da) | Protein pI |
|---|---|---|---|---|---|---|---|
| 1 | Phosphopyruvate hydratase | 142.37 | 8 | 78 | 29 | 46802 | 6.16 |
| 2 | Phosphoglycerate kinase | 137.35 | 9 | 29 | 33 | 44738 | 7.11 |
| 3 | Pyruvate kinase | 115.12 | 7 | 9 | 28 | 54599 | 8.00 |
| 4 | Pyruvate decarboxylase | 112.05 | 7 | 8 | 21 | 61495 | 5.80 |
| 5 | Reading Frame | 84.38 | 4 | 114 | 28 | 35731 | 6.46 |
| 6 | Heat-Shock Protein 26 kD | 84.12 | 5 | 9 | 30 | 23879 | 5.32 |
| 7 | Aldehyde dehydrogenase | 72.17 | 5 | 6 | 18 | 56723 | 6.31 |
| 8 | Hexokinase A | 71.71 | 4 | 6 | 14 | 53738 | 5.28 |
| 9 | Alcohol dehydrogenase I | 57.18 | 4 | 12 | 16 | 36823 | 6.26 |
| 10 | Triosephosphate isomerase | 49.69 | 4 | 4 | 30 | 26795 | 5.74 |
| 11 | Ketol-acid reductoisomerase | 46.82 | 3 | 3 | 12 | 44368 | 9.11 |
| 12 | Phosphoglycerate mutase | 44.71 | 3 | 4 | 19 | 27608 | 8.81 |
| 13 | 60S Ribosomal Protein L4-B | 38.32 | 3 | 3 | 17 | 39062 | 10.64 |
| 14 | Glucose kinase | 35.38 | 2 | 2 | 7 | 55377 | 5.80 |
| 15 | Hexokinase PII | 33.32 | 2 | 2 | 14 | 27485 | 5.19 |
| 16 | Pyrophosphatase | 32.77 | 3 | 3 | 11 | 32315 | 5.36 |
| 17 | 60S Ribosomal Protein L5 | 31.08 | 2 | 2 | 13 | 33743 | 6.36 |
| 18 | BMH1 | 29.17 | 2 | 4 | 15 | 30176 | 4.87 |
| 19 | Translation Elongation Factor eEF-1 | 28.39 | 2 | 10 | 7 | 50032 | 9.14 |
| 20 | Citrate (si)-synthase | 25.16 | 2 | 2 | 5 | 53360 | 8.23 |
| 21 | Superoxide dismutase | 24.66 | 2 | 2 | 27 | 15854 | 5.62 |
| 120 | Polyadenylate-binding protein | 9.10 | 1 | 1 | 1 | 64344 | 5.71 |
| 121 | Ribosomal Protein S15 | 9.06 | 1 | 1 | 13 | 16001 | 10.70 |
| 122 | Ribosomal Protein S14 | 9.03 | 1 | 1 | 7 | 14649 | 10.54 |

**Table 2**
**21 top score proteins identified from a yeast cell lysate as well as the three proteins identified with the lowest confident score.**

| Method | Online 2D LC with injected salt steps | Online 2D LC with pumped semi-continuous salt gradient | Off-line 2D LC with pumped linear salt gradient |
|---|---|---|---|
| **Identified Proteins** | 101 | 122 | 144 |
| **Assigned Peptides** | 179 | 207 | 269 |

**Table 3**
**Comparison of the performance of different 2D LC methodologies.**

To show the full competitiveness of this new methodology the results obtained by the semi-continuous salt gradient approach was compared to the conventionally used injected salt step gradient as well as to the high resolution off-line 2D methodology, which works with a linear continuous salt gradient elution in the first dimension[9]. For this comparison the same sample, the same columns and comparable salt steps were used. The results of these experiments are outlined in table 4. The number of identified proteins as well as the number of assigned peptides increases from the injected step gradient approach to the semi-continuous gradient approach. It is also evident that the off-line methodology is superior to the on-line variants although its related higher effort. However, these results demonstrate clearly the improvement, which can be realized with slight modifications of the system as described for the injected step gradient approach[2] to gain better results.

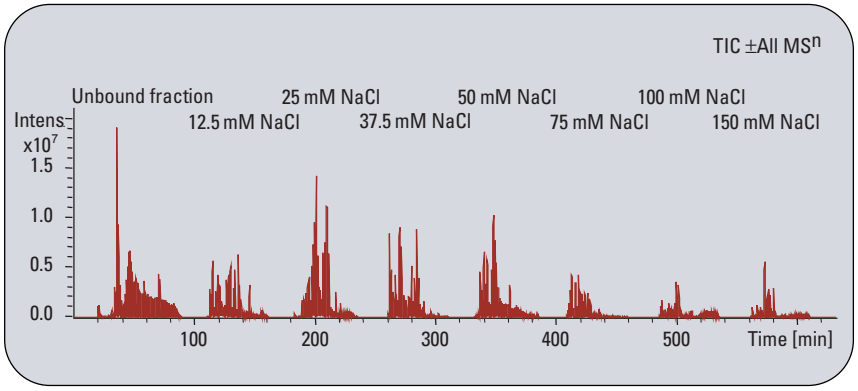


**Figure 7**
**MS/MS spectra acquired during all RP separations**

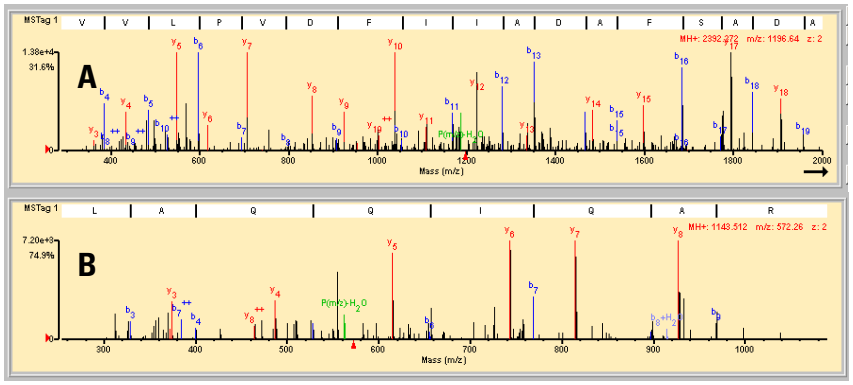


**Figure 8**
**Fragmentation pattern of tryptic peptides from phosphoglycerat kinase (A) and polyadenylat-binding protein (B)**

| Method | Online 2D LC with injected salt steps | Online 2D LC with pumped semi-continuous salt gradient | Off-line 2D LC with pumped linear salt gradient |
|---|---|---|---|
| **SCX Resolution** | low | high | very high |
| **Automation** | high | high | medium |
| **Complexity of set-up** | medium | high | medium |
| **Investment** | low | medium | high |
| **Effort** | medium | medium | high |
| **Access to SCX fractions** | no | no | yes |
| **Flexibility** | low | low | high |

**Table 4**
**Overview comparison of the semi-continuous gradient on-line 2D LC methodology to the injected step gradient on-line 2D LC methodology and to the off-line 2D methodology**

## Conclusion

This Application Note demonstrates that the performance of the classical approach of two-dimensional LC/MS for protein identification, which works with injected salt solution plugs of increasing concentration to elute peptides of digested proteome samples from the first dimension SCX column can be improved significantly. This improvement is mainly due to the applied semi-continuous salt solution gradient, which keeps the SCX column in an equilibrium state during peptide elution in the first dimension. In this equilibrium state the peptides are eluted much more concentrated and therefore it is easier to detect peptides from lower abundant proteins. To utilize this effect only minor hardware investment is necessary, mainly a precise second pump and a micro 10-port valve with two enrichment columns. Table 4 shows an overview comparison of the semi-continuous gradient on-line 2D LC methodology to the injected step gradient on-line 2D LC methodology and to the off-line 2D methodology. The described methodology allows to analyze real life

proteomics samples with high complexity in a highly automated manner with maximized results. Moreover, this approach has the potential for further improvement by adapting the method to a real continuous salt gradient for the first dimension SCX chromatography.

## References


1.
"Automated LC-LC—MS-MS platform using binary ion-exchange and gradient reversed-phase chromatography for improved proteomic analyses.", Davis MT, Beierle J, Bures ET, McGinley et al., *J. Chromatogr. B 752: 281-291*, **2001.**

2.
a) "Two-dimensional nano-liquid chromatography-mass spectrometry system for applications in proteomics", Nägele E, Vollmer M, Hörth P., *J. Chromatogr. A 1009: 197-205*, **2003.**
b) "Two-dimensional nano-LC/MS for complex proteomics application.", *Agilent Application Note*, *Publication number 5988-9287EN*, **2003.**

3.
"Large-scale analysis of the yeast proteome by multidimensional protein identification technology.", Washburn MP, Wolters D, Yates JR., *Nat. Biotechnol. 19: 242-247*, **2001.**

4.
"A proteomic view of the *Plasmodium falciparum* life cycle.", Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, et.al., *Nature 419: 520-526*, **2002.**

5.
a) "Differential Proteome analysis: Two-dimensional nano LC/MS of *E. Coli* proteome grown on different carbon sources.", *Vollmer M*, *Nägele E*, *Hörth P.*, *J. Biomol. Tech. 14: 128-135*, **2003.**
b) "Two-dimensional nano LC/MS of *E. Coli* proteome grown on different carbon sources", *Agilent Application Note*, *Publication number 5988-9862EN*, **2003.**

6.
a) "Proteome Profiling of E. coli: Effect of Heat-Shock Conditions on Protein Expression", Hörth P, Nägele E, Vollmer M., *LC/GC Europe*, *Sept.*, **2003.**
b) "Proteome profiling of *E. coli*: Effect of heat-shock conditions on protein expression.", *Agilent Application Note*, *Publication number 5988-8629EN*, **2003.**

7.
"Agilent Nanoflow Proteomics Solution", *Agilent Product Brochure*, *Publication Number 5988-3612EN*, **2002.**

8.
"Agilent Spectrum Mill MS Proteomics Workbench", *Agilent Product Brochure*, *Publication Number 5988-9443EN*, **2003.**

9.
"Improved protein identification: Off-line multidimensional LC/MS as an effective tool for proteomics research.", *Agilent Application Note*, *Publication number 5988-9913EN*, **2003.**



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